PLANT ANTICANCER AGENTS. XIX. CONSTITUENTS OF *AQUILARIA MALACCENSIS*[1]

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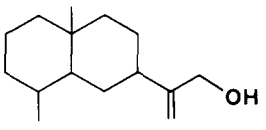
ABSTRACT.—The stem bark of the Thai tree *Aquilaria malaccensis* (Thymelaeaceae) has afforded 1,3-dibehenyl-2-ferulyl glyceride (**3**), which is novel, and 12-*O*-*n*-deca-2,4,6-trienoylphorbol-13-acetate (**4**). The structures of these cytotoxic compounds were elucidated by their spectral and chemical parameters.

Aquilaria malaccensis Lamk. (syn. *A. agallocha* Roxb.)[2] is distributed in the areas of Bengal and Assam in India, in Burma, Thailand, the Malay Peninsula, Borneo, and the Philippines. The sample used in the presently described study originated in Thailand where the common names are Kaa-yuu kaa-huu and Mai hom.

There are no ethnomedical properties reported, but an alcoholic extract has been reported to exhibit mild cardiotonic activity (3). The infected (*Cytosphaera*



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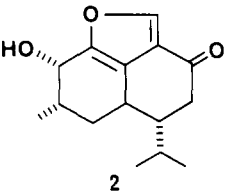

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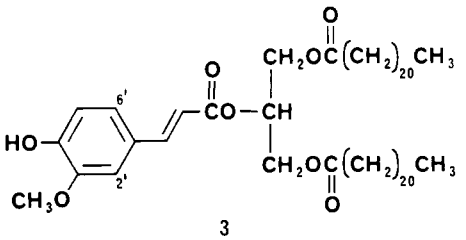

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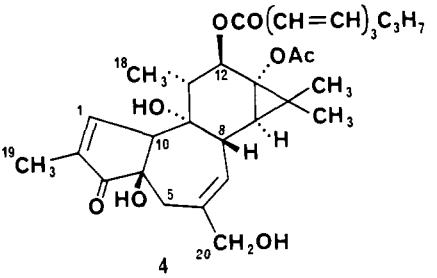

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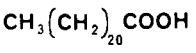

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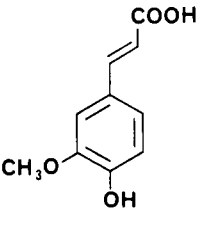

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[1]For the previous paper in this series see reference 1.

[2]*A. malaccensis* Lamk. is also synonymous with *Agallochum secundarium* Rumph., *Agallochum coinamense* Rumph., *Agallochum malaccense* Rumph., *Aquilaria ovata* Cava., *Aquilaria secundaria* D.C., *Agallochum malaccense* (Lamk.) Kuntze and *Aquilariella malaccensis* (Lamk.) Van Tiegh. (2).

mangiferae Died.) wood of this plant is the prime source of the perfume agar (4–10), and the pulp has also been used in paper manufacture (11).

Relatively little phytochemical work has been conducted on the genus, although as a result of prior work, an unprecedented situation now exists where two different compounds from *A. malaccensis*, namely **1** and **2**, have each been assigned the same name, agarol (3, 12), even though they have substantially different structures. It is recommended that the name agarol be reserved for **1**, and that **2** be known as 8β H-dihydrogmelofuran.

Our attention was turned to *A. malaccensis* when an aqueous ethanolic extract of the stem bark and stem was found to display significant activity in the Eagles' carcinoma of the nasopharynx (KB) test system.[3] Although this activity was confirmed in a recollection, more substantial activity was demonstrated in the P-388 lymphocytic leukemia system *in vitro*. It was therefore the latter test system which was used in the bioactivity-directed fractionation of the plant extract, which led to the isolation of the principal active constituents **3** and **4**.

EXPERIMENTAL[4]

PLANT MATERIAL.—The plant material was collected in Thailand in October 1978, and identified as *Aquilaria malaccensis* Lamk. by the Economic Botany Laboratory, Science and Education Administration, BARC-East, U.S.D.A., Beltsville, Maryland.

EXTRACTION AND FRACTIONATION.—Air dried and milled stem bark of *A. malaccensis* (27.2 kg) was successively extracted with petroleum ether (bp 60–90°) and chloroform. Concentration of the extracts *in vacuo* afforded residues weighing 122 g and 140 g respectively.

BIOASSAY OF THE CRUDE EXTRACTS.—The petroleum ether and chloroform extracts displayed ED_{50} 0.35 and 0.41 μg/ml respectively in the P-388 lymphocytic leukemia system in cell culture.

SEPARATION OF THE PETROLEUM ETHER EXTRACT.—A portion of the petroleum ether extract (1.7 g) was chromatographed on a column of Florisil[5] (100–200 mesh) (40 g) packed in petroleum

ISOLATION AND IDENTIFICATION 1,3-DIBEHENYL-2-FERULYL GLYCERIDE (**3**).—Fraction 7 (520 mg) on crystallization from methylene chloride-*n*-hexane (1:9) yielded 1,3-dibehenyl-2-ferulyl glyceride (**3**) as white needles (210 mg, 0.55%), mp 84–5°; ir, νmax (KBr) 3400, 1732, 1706, 1629, 1512, 1385, 1368, 1262, 1024, 983, 838, 827 and 712 cm^{-1}: uv, λmax (MeOH) 218 (log ϵ 4.06), 238 (3.99), 298 (sh) (4.09), and 327 nm (4.17); nmr, δ ($CDCl_3$) 0.87 (6H, t, J=4.6 Hz, 2 x terminal $-CH_3$), 1.26 (76H, broad m, CH_2 aliphatic), 2.33 (4H, t, J=6.7 Hz, 2 x $-OCOCH_2-$), 3.92 (3H, s, 3'-OCH_3), 4.30 (4H, m, 1- and 3-H_2), 5.35 (1H, quintet, J=4.7 Hz, 2-H), 6.04 (1H, d, J=15.8 Hz, vinylic-H), 6.88 (1H, d, J=8.3 Hz, 5'-H), 7.02 (1H, d, J=1.5 Hz, 2'-H), 7.08 (1H, dd, J=1.5, 8.3 Hz, 6'-H) and 7.63 (1H, d, J=15.8 Hz, vinylic H).

HYDROLYSIS OF 1,3-DIBEHENYL-2-FERULYL GLYCERIDE (**3**).—Hydrolysis of 1,3-dibehenyl-2-ferulyl glyceride (**3**, 99 mg) with 1% KOH in 90% ethanol-water (20 ml) under reflux for 2.5 hrs and acidification followed by extraction with chloroform afforded a gum (85 mg). Separation on silica gel[6] tlc eluting with 10% methanol-chloroform afforded two acidic components.

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[3]The extracts, fractions and compounds were tested under the auspices of the Drug Research and Development Program of the National Cancer Institute (13). An isolate is considered active if it shows an $ED_{50} \leq 4$ μg/ml in the KB or P-388 cell culture *in vitro*, and a T/C $\geq$125% *in vivo*.

[4]Melting points were determined on a Kofler hot plate and are uncorrected. The uv spectra were obtained with a Beckman model DB-G spectrophotometer. The ir spectra were obtained with a Beckman model 18-A spectrophotometer with polystyrene calibration at 1601 cm^{-1}; absorption bands are recorded in wave numbers (cm^{-1}). Pmr spectra were recorded in $CDCl_3$ with a Varian T-60A instrument operating at 60 MHz with a Nicolet Model TT-7 Fourier Transform attachment. Tetramethylsilane was used as an internal standard and chemical shifts are reported in δ (ppm). Mass spectra were obtained with a Varian MAT-112S double focusing spectrometer operating at 70 eV.

[5]Fisher Scientific Co. Chicago, IL, U.S.A.
ether. Elution with petroleum ether-ether (19:1), petroleum ether-ether (4:1), petroleum ether-ether (1:1), chloroform and chloroform-methanol, gave fractions 1, 2-3, 4-5, 6-8 and 9 respectively.

[6]E. Merck, Darmstadt, West Germany.

The less polar component, on crystallization from *n*-hexane, afforded white crystals of behenic acid (*n*-docosanoic acid) (**5**, 45 mg), mp 81–2°, [lit. (14), mp 82°], ir, νmax (KBr) 3100, 2960, 2920, 2850, 1705, 1465, 1455, 1425, 1395, 1295, 1110, 720 and 710 cm^{-1}; nmr δ ($CDCl_3$) 0.87 (3H, t, J=4.9 Hz, terminal CH_3), 1.26 (38H, broad m, CH_2 groups), and 2.35 (2H, t, J=6.7 Hz, $-CH_2CO_2H$); ms, *m/z* 340 (M^+, 31%), 313 (3), 312 (14), 297 (7), 284 (5), 269 (5), 256 (5), 254 (4), 241 (10), 227 (6), 199 (6), 185 (14), 171 (9), 157 (7), 143 (7), 129 (34), 115 (10), 101 (7), 87 (17), 73 (68), 59 (62), 56 (85), 54 (77) and 43 (100).

The more polar component, on crystallization from *n*-hexane-methylene-chloride (9:1), afforded white crystals of ferulic acid (**6**, 12 mg), mp 169°, [lit. (15), mp 169°]; uv, λmax (MeOH) 220 (log ε 4.24), 232 (4.20), 296 (sh) (4.32), and 318 nm (4.36), λmax (MeOH–KOH) 232 (log ε 4.20), 304 (sh) (4.16) and 348 nm (4.39); ir, νmax (KBr) 3440, 3020, 2970, 2850, 2520, 1685, 1657, 1612, 1590, 1505, 1457, 1420, 1400, 1340, 1310, 1270, 1225, 1192, 1152, 1108, 1025, 960, 920, 840, 790 740 cm^{-1}; nmr, δ ($CDCl_3$) 3.92 (3H, s, 3-OCH_3), 6.34 (1H, dd, J=15.4 Hz, vinylic –H), 6.85 (1H, d, J=8.4 Hz, 5H), 7.15 (1H, dd, J=1.5, 8.4 Hz, 6-H), 7.30 (1H, d, J=1.5 Hz, 2-H), and 7.60 (1H, d, J=15.4 Hz, vinylic –H); ms, *m/z* 196 (M^+, 48%), 195 (100), 194 (70), 193 (6), 181 (12), 180 (27), 179 (20), 178 (11), 177 (11), 161 (12), 152 (11), 148 (8), 146 (4), 145 (15), 135 (11), 134 (29), 133 (43), 124 (9), 117 (14), 106 (13), 105 (21), 89 (20), 79 (13), 78 (24), 77 (27), 69 (13), 63 (16), 54 (12), 53 (14), 51 (26) and 28 (63).

Isolation of 12-*O*-*n*-deca-2,4,6-trienoylphorbol-13-acetate (**4**).—The petroleum ether fraction (116 g) was chromatographed on a column of silica gel[6] (2.4 kg) packed in petroleum ether. Elution with petroleum ether, petroleum ether-chloroform (9:1) and chloroform afforded fractions 1–2, 3–4 and 5–10 respectively. The most active (fraction 10) (30 g, ED_{50} 0.038 μg/ml) was chromatographed on silica gel[6] (600 g) packed in chloroform. Elution with chloroform and chloroform-methanol (9:1) gave fractions 1–7 and 8–15 respectively. The most active fraction (fraction 13) (350 mg, ED_{50} 0.034 μg/ml) was rechromatographed on silica gel[6] (20 g) packed in chloroform. Elution with chloroform, chloroform-methanol (99.5:0.5), chloroform-methanol (99:1) and chloroform-methanol (98:2) afforded fractions 1, 2, 3 and 4 respectively. Once again the most active fraction (fraction 3) (80 mg, ED_{50} 0.16 μg/ml) was separated by silica gel tlc eluted twice with ethyl acetate. A uv visible band was separated, extracted with chloroform, and concentrated to afford 12-*O*-*n*-deca-2,4,6-trienoylphorbol-13-acetate (**4**) as a gum (42 mg, 0.0016%), $[\alpha]^{25}D$ −15.3° (c 0.2, $CHCl_3$) [lit. (16) $[\alpha]^{25}D$ −19° ($CHCl_3$)], Rf 0.41 ($CHCl_3$-MeOH, 24:1); ir, νmax (thin film) 3420, 2975, 2940, 2873, 1737, 1726, 1715, 1622, 1460, 1378, 1325, 1260, 1127, 1005 and 758 cm^{-1}; uv, λmax (MeOH) 232 (sh) (log ε 4.12) and 307 nm (4.53); nmr, δ ($CDCl_3$) 0.87 (3H, d, J=6 Hz, 18-CH_3), 0.90 (3H, t, J=6 Hz, terminal CH_3), 1.06 (1H, d, J=5 Hz, 14-H), 1.22 and 1.26 (6H, two s, 16,-17-CH_3), 1.73 (3H, bd s, 19-CH_3), 2.09 (3H, s, 13-$OCOCH_3$), 2.54 (2H, s, 5-H_2), 3.24 (2H, m, 8-H and 10-H), 4.00 (2H, s, 20-H_2), 5.46 (1H, d, J=10.4 Hz, 12-H), 5.5–7.5 (8H, m, vinylic-H), and 7.57 (1H, bd s, 1-H); ms, *m/z* M^+ not observed, 389 (2%), 328 (5), 310 (6), 309 (2), 222 (12), 221 (5), 220 (3), 167 (9), 166 (5), 149 (6), 123 (12), 121 (12), 113 (9), 109 (30), 107 (19), 79 (21), 69 (48), 54 (27), 43 (96) and 28 (100). These data are in accord with those reported previously (16, 17) for **4**. Comparison with spectral data generously provided by Professor Mitsui of Okayama University confirmed the identity.

Biological activities of the isolates.—1,3-Dibehenyl-2-ferulyl glyceride (**3**, NSC-332563) displayed ED_{50} 0.8 μg/ml against the P-388 test system *in vitro*, but was inactive against the same system *in vivo*. 12-*O*-*n*-Deca-2,4,6-trienoylphorbol-13-acetate (**4**, NSC-336793) displayed ED_{50} 0.0022 μg/ml in the P-388 test system *in vitro*.

Structure elucidation 1,3-dibehenyl-2-ferulyl glyceride (**3**).—1,3-Dibehenyl-2-ferulyl glyceride showed no optical rotation in either chloroform or pyridine, indicating either the absence of asymetric centers or the presence of a *meso* compound. The mass spectrum displayed a molecular ion at *m/z* 912 analyzed for $C_{57}H_{100}O_6$. A characteristic green coloration was observed with $FeCl_3$ solution for the presence of a phenolic group which was confirmed when the uv maximum at 327 nm was shifted in base, indicating the presence of *p*-hydroxy cinnamoyl group. Strong absorptions were observed in the ir spectrum at 3400 cm^{-1} (phenolic group), at 1732 cm^{-1} (ester group) and 1706 cm^{-1} (α,β-unsaturated ester group) and at 1629 cm^{-1} for a disubstituted olefinic moiety. The proton nmr spectrum indicated the presence of an exchangeable OH at 6.00 ppm, an aromatic methoxy group at 3.92 ppm, a 1,2,4-trisubstituted aromatic system, and two *trans*-coupled olefinic protons. These signals suggested the presence of a 3-methoxy-4-hydroxy cinnamoyl group. In addition, the pmr spectrum showed a quintet at 5.35 ppm for a single proton and four-proton multiplet at 4.30 ppm characteristic (18) of a symmetrically substituted glycerol, and a four proton "triplet" at 2.33 ppm for two CH_2 groups adjacent to an ester moiety. A broad singlet for 76 protons and a six proton triplet (0.87 ppm) indicated the esterifying groups to be long chain saturated acids.

Base hydrolysis of the isolate afforded only ferulic and behenic acids in a 1:2 molar ratio, whose structures were confirmed by their spectral properties. A combination of the above data indicated the structure of the isolate to be 1,3-dibehenyl-2-ferulyl glyceride (**3**).

DISCUSSION

This is the first reported isolation of **3** and the first demonstration that compounds of this structure class display *in vitro* cytotoxic activity.

The compound 12-*O*-*n*-deca-2,4,6-trienoylphorbol-13-acetate (**4**) was first isolated from *Sapium japonicum* (Sieb & Zucc.) Pax. et Hoffm. (Euphorbiaceae) (16) and *Euphorbia tirucalli* L. (Euphorbiaceae) (17). Tigliane and daphnane esters are well recognized as being cytotoxic (19, 20), and as more examples are disclosed it may well be possible to develop parameters for the potentiation of their activity.

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